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Characterization of the Lysogeny DNA Module from the Temperate

Anne Bruttin,[1] Frank Desiere,[1] Sacha Lucchini, Sophie Foley, and Harald Brüssow[2]

Nestlé Research Centre, Nestec Ltd., Vers-chez-les-Blanc, CH-1000 Lausanne 26, Switzerland

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Phage ϕSfi21, the only temperate *Streptococcus thermophilus* phage from our phage collection, showed extensive DNA homology with virulent phages from lytic group I. Southern blot hybridizations demonstrated that the ϕSfi21-specific DNA was clustered in an approximately 6.6-kb-long region, the putative lysogeny module. Sequence analysis and database research identified an integrase within this module; orf 203 with homology to an anonymous orf 258 from the temperate lactococcal phage BK5-T; orf 127 and orf 122 with weak homology to the N- and C-terminal parts, respectively, of the cI-like repressor from lactococcal phages Tuc2009 and BK5-T; orf 75 with homology to a repressor protein from lambdoid phage 434 and an anti-repressor *ant* with homology to phage P1. The molecular arrangement of the predicted orfs in phage ϕSfi21 was very similar to that of the lactococcal phage BK5-T. The transition from ϕSfi21-specific DNA into DNA shared with virulent phages was abrupt and flanked at one side by notable DNA repeats. Sequence analysis identified a holin protein to the left of the lysogeny module. A site-specific deletion of 2.4 kb, which reproducibly transformed ϕSfi21 into a lytic phage, was localized in the lysogeny module. It was flanked at both sides by conspicuous DNA repeats. One repeat region reflected the DNA around the *attP* site, while the other reflected the putative genetic switch region between repressor and anti-repressor genes. *S. thermophilus* host Sfi1 transformed with a plasmid containing *int* and orf 203 showed resistance to superinfection by heterologous phages, but not by the homologous ϕSfi21. Part of the *int* gene could be deleted without loss of this activity, while a deletion in orf 203 resulted in loss of the phage resistance. We speculate on the possibility of a bipartite immunity system for the control of lysogeny in ϕSfi21.

INTRODUCTION

An understanding of the evolution of bacteriophages is of both theoretical and practical value. We decided to address some of the questions on phage evolution using phages of *Streptococcus thermophilus,* a gram-positive lactic acid bacterium used in milk fermentation (Mercenier, 1990). Phage infection has always been a major problem in industrial fermentation, especially in the dairy industry (Peitersen, 1991). Contamination by virulent phages may result in the lysis of bacterial starter strains, causing slow fermentation or even complete starter failure with consequent loss of the product. *S. thermophilus* phage control measures have been hampered by the large variability of phages encountered in the environment (Benbadis *et al.,* 1990; Brüssow *et al.,* 1994a; Neve *et al.,* 1989; Prevots *et al.,* 1989) and the lack of knowledge of their genome organization (Brüssow and Bruttin, 1995). Analysis of phages from our phage collection covering 30 years of industrial fermentation (Brüssow *et al.,* 1994a) and from an ecological survey (Bruttin *et al.,* 1997) indicated that very many, perhaps several hundred, distinct *S. thermophilus* phages may exist in the environment.

From work with phage λ it has been inferred that the various races of phage λ are related by recombination events. Careful analysis of lambdoid phages by sequencing and heteroduplex analysis revealed that homologous and heterologous genome segments were interspersed (Campbell and Botstein, 1983). Where sequence data locate the transitions between homology and heterology on the genetic map, the transition points frequently lie strikingly close to the boundaries of functional segments of the genome (Casjens *et al.,* 1992). This observation gave rise to the concept of phage evolution by exchange of modules, where a module implies a stretch of genes of related function (Susskind and Botstein, 1978).

Some observations of *S. thermophilus* phages can be interpreted within the module exchange hypothesis (Brüssow *et al.,* 1994b). Systematically, dot blot hybridization studies (Brüssow and Bruttin, 1995) showed a close genetic relationship between temperate phage Sfi21 and lytic group I phages of *S. thermophilus* in that the temperate phage Sfi21 differed from these lytic phages only in the possession of a single contiguous DNA fragment. The ϕSfi21-specific DNA is flanked at both sides by conserved DNA sequences of lytic group I phages (Brüssow and Bruttin, 1995). Furthermore, the ϕSfi21-specific DNA fragment was also found in another temperate *S. ther-*

[1] Both authors contributed equally to this work

[2] To whom correspondence and reprint requests should be addressed. Fax: 0041 21 785 8925. E-mail: Harald.Bruessow(a)CHLSNR.NESTRD.CH.

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mophilus phage, TP-J34 (Neve *et al.,* 1997), but, in contrast, the rest of the genome of this phage showed little or no homology with φSfi21 (Brüssow and Bruttin, 1995). The temperate phage Sfi21 could thus have been created through the acquisition of a hypothetical lysogeny module by the genome of a lytic phage.

Interestingly, upon serial passages of phage φSfi21 we repetitively observed a 2.4-kb deletion which transformed the temperate phage into a lytic phage (Bruttin and Brüssow, 1996). As the deletions occurred at exactly the same nucleotide positions in several independent mutants, one may suspect an enzymatic mechanism behind this deletion process. We envisioned two hypotheses. First, the deletion process is the result of a hypothetical site-specific recombinase responsible for exchanging genome segments in the process of module shuffling. Alternatively, the deletion process resulted from an error of the phage-encoded integrase which used the phage *attP* site and, instead of the bacterial *attB* site, a secondary *att* site on the phage DNA for prophage integration. Such an accidental integration process has already been demonstrated for phage λ (Davis and Parkinson, 1971).

To settle this question and to further our understanding of the genetic organization of the lysogeny module from *S. thermophilus* phages we decided to sequence the φSfi21-specific DNA.

MATERIALS AND METHODS

Phages, strains, and media

Phage Sfi21 was propagated on *S. thermophilus* strain Sfi1 in lactose M17 broth as described previously (Bruttin and Brüssow, 1996; Brüssow and Bruttin, 1995). *Escherichia coli* strain JM 101 (Stratagene) was grown in LB broth or on LB broth solidified with 1.5% (w/v) agar. Ampicillin, IPTG and *Xga*I (all from Sigma) were used at concentrations of 100 μg/ml, 1 m*M,* and 0.002% (w/v), respectively. Lytic phages from our phage collection (Brüssow *et al.,* 1994a) were used for comparative Southern blot hybridization.

DNA techniques

Phage purification, DNA extraction, agarose gel electrophoresis, Southern blot hybridization, and labeling of plasmid DNA were done as described previously (Bruttin and Brüssow, 1996; Brüssow and Bruttin, 1995). General DNA techniques were performed as described by Sambrook *et al.* (1989). Plasmid DNA was isolated using Qiagen midi-plasmid isolation columns. Restriction enzymes were obtained from Boehringer Mannheim and used according to the supplier's instructions.

Cloning

Phage Sfi21 DNA was cut with restriction enzymes *Eco*RI, *Hin*dIII, *Nsi*I, or *Xba*I and cloned into pUC19 vector or the *E. coli*/lactococcal/streptococcal shuttle vector pNZ124 (Platteeuw *et al.,* 1994). In addition, total phage Sfi21 DNA was cut with restriction enzyme Sau3A and used for shotgun cloning into pUC19.

Sequencing

DNA sequencing was started with the universal forward and reverse primers of pUC19 or pNZ124 and continued with synthetic oligonucleotide (17-mer) primers (Microsynth, Switzerland). Cloned DNA was sequenced on both strands by the Sanger method of dideoxy-mediated chain termination using the *fmol* DNA Sequencing System of Promega (Madison,WI). The sequencing primers were end-labeled using [γ-^{33}P]ATP according to the manufacturer's protocol. The thermal cycler (Perkin–Elmer) was programmed at 30 cycles of −95° for 30 sec, 50° for 30 sec, and 72° for 1 min.

pUC19 clones containing *Sau*3A-digested phage Sfi21 DNA were sequenced by the Amersham Labstation sequencing kit based on Thermo Sequenase labeled primer cycle sequencing with 7-deaza-dGTP(RPN2437). Sequencing was done on a Licor 6000L automated sequencer with fluorescence-labeled universal reverse and forward pUC19 primers. No *Sau*3A clones were obtained for the middle of the *Xba*I fragment 3.

PCR

PCR was used to prepare a template for sequencing the middle part of *Xba*I fragment 3 that could not be obtained by cloning. PCR products were prepared using the indicated synthetic oligonucleotide pair (5′-3′ primer 1, CGTTTCAAGGCGTGGGC, and primer 2, CGCTCGCGCTTTAGGTT), purified phage DNA and Super Taq Polymerase (Stehelin, Basel, Switzerland). PCR products were purified using the QIAquick-spin PCR Purification kit.

Sequence analysis

The Genetics Computer Group sequence analysis package (University of Wisconsin) was used to assemble and analyze the sequences. Nucleotide (nt) and predicted amino acid (aa) sequences were compared to those in the databases (GenBank, Release 97.0; EMBL (Abridged), Release 48.0; PIR-Protein, Release 50.0; SWISS-PROT, Release 33.0; PROSITE, Release 13.0) using the FastA (Pearson and Lipman, 1988) and BLAST (Altschul *et al.,* 1990) programs. Sequence alignments were performed using the CLUSTALW 1.6 method (Thompson *et al.,* 1994) and the BLOCKMAKER program (Henikoff *et al.,* 1995). The integrase tree was built with the program Alignment of GeneWorks (V2.0) from IntelliGenetics Inc.

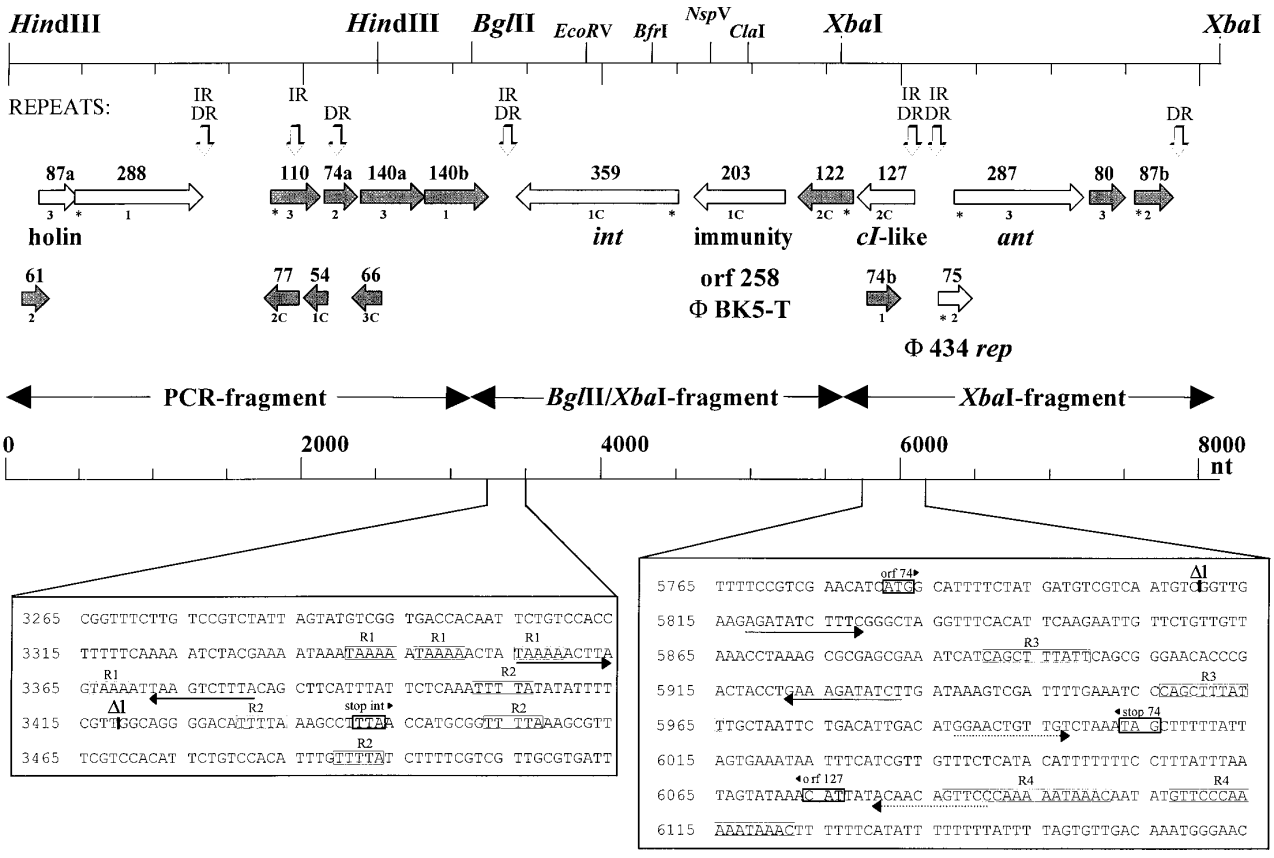


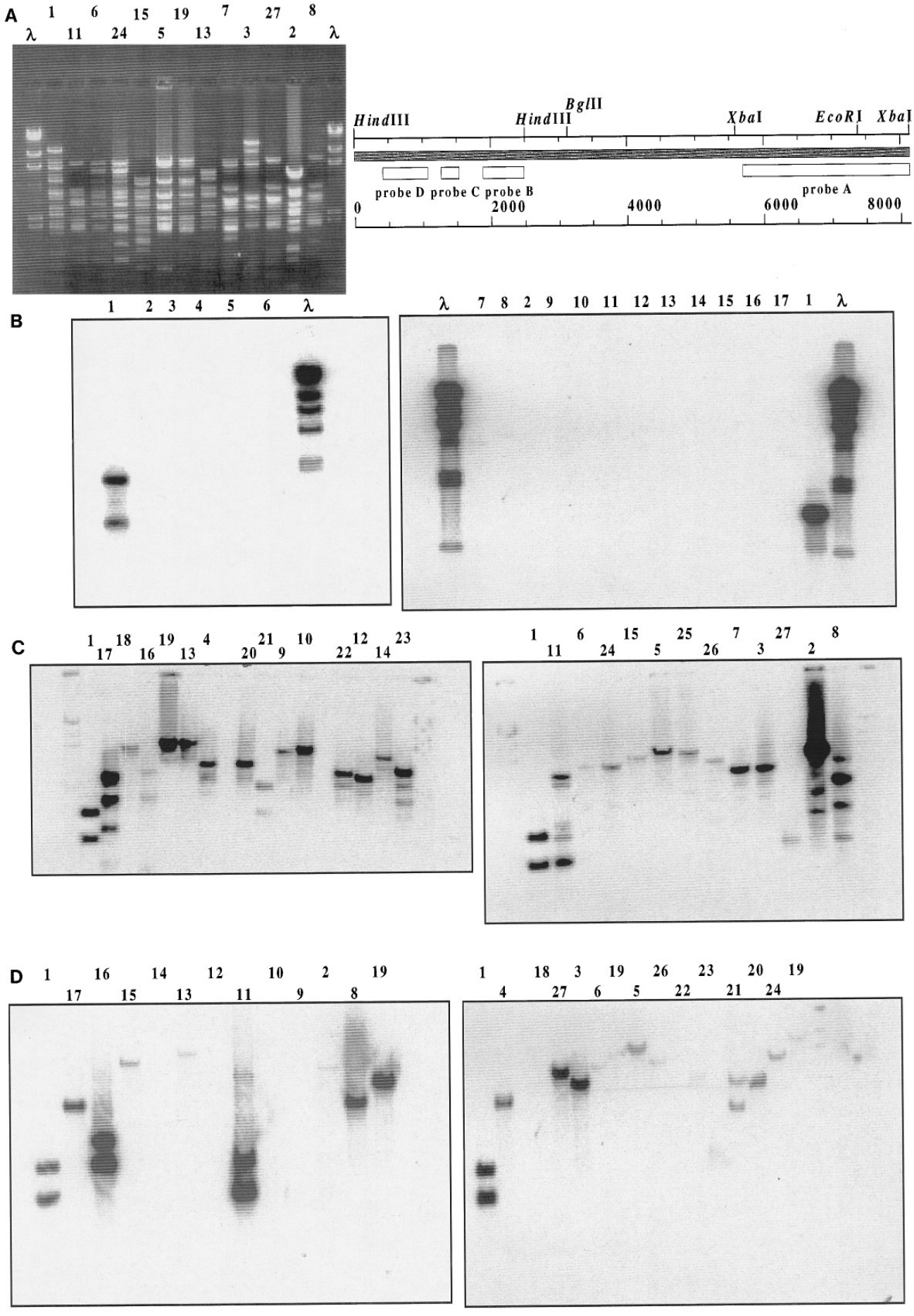
FIG. 1. Prediction of open reading frames (orfs) longer than 50 codons for the investigated phage Sfi21 DNA sequence. The length of the predicted proteins in amino acids was noted above the arrows; predicted proteins with identical length were differentiated with a or b annotations. Reading frame 1, 2, or 3 and location on the complementary strand (C) were indicated below the arrows. Orfs showing matches in the database searches were lightly shaded and were annotated with genetic symbols. * Indicates standard ribosomal binding site. The upper line gives the position of some relevant restriction sites, the lower line the nucleotide position mentioned in the text. Direct repeats (DR) and inverted repeats (IR) were marked with arrows in the figure and were shaded in the sequences shown for the two indicated regions. The positions of the left and right deletion sites on the spontaneous phage deletion mutant are marked by Δ1. This sequence is available from the EMBL Database under Accession No. X95646.

RESULTS

Cloning of DNA fragments affected by the 2.4-kb deletion

Phage Sfi21 DNA was cut with a number of restriction enzymes which had been mapped and the resulting fragments were cloned into plasmid vectors. No clones were obtained for the left and right end of the genome containing the *cos* sites and a central part of the genome in which the type 2 deletion was localized (Bruttin and Brüssow, 1996). To bridge the central gap, sequence information from the left and rightmost available clones bordering the gap were obtained and the unclonable DNA fragment was amplified by PCR. Partial sequencing of the PCR product identified a *Bgl*II site in the middle of the unclonable DNA fragment. A *Bgl*II/*Xba*I fragment of the phage DNA, representing the right half of this unclonable area and covering the DNA deleted in the type 2 deletion mutants (Bruttin and Brüssow, 1996), was then successfully cloned into the vector pNZ124, yielding plasmid pPX3R (for *Pvu*II/*Xba*I fragment 3, right part) and sequenced. In contrast, the 3.1 kb of phage DNA to the left of the *Bgl*II restriction site still could not be cloned and therefore a PCR product was sequenced. The localization of the holin gene in this DNA region (see below) might explain why this DNA was unclonable.

FIG. 2. Definition of the lysogeny module in ϕSfi21 by Southern blot hybridization against a panel of 26 virulent phages. (A) Left, a representative restriction pattern (*Bam*HI/*Hin*dIII) of *S. thermophilus* phages (listed below) as revealed by ethidium bromide fluorescence. Right, Localization of the hybridization probes A to D on the map presented in Fig. 1. (B to D) Southern blots of the *Eco*RI/*Xba*I (B, left) and *Eco*RV (B, right; C and D) digests of the indicated phages hybridized with probes A (B, left), B (B, right), C (C) and D (D). Phage (lane) coding: ϕSfi21 (1), Sfi3J (7), Sfi18 (11); ϕS5 (15), Sfi19 (2), S69 (17), S94 (20), S96 (21); ϕST3 (8), ST12 (23), ST44A (3), ST128 (16), ST130 (27); ϕB (5), C (13), E (18), F (26), H (6), I (19), L (24), M (4); ϕL-A3 (9), L-A4 (10), L-A9 (22), L-A12 (12),L-F1 (14).


A
1 6 15 19 7 27 8
λ 11 24 5 13 3 2 λ
HindIII
HindIII
BglII
XbaI
EcoRI
XbaI
probe D
probe C
probe B
probe A
0
2000
4000
6000
8000
B
1 2 3 4 5 6 λ
λ 7 8 2 9 10 11 12 13 14 15 16 17 1 λ
C
1 18 19 4 21 10 12 23
17 16 13 20 9 22 14
1 6 15 25 7 27 8
11 24 5 26 3 2
D
1 16 14 12 10 2 19
17 15 13 11 9 8
1 18 3 19 26 23 20 19
4 27 6 5 22 21 24

The deletion is flanked by DNA repeats

PCR using primers located to the right and left side of the deletion yielded an approximately 6-kb DNA product for the wild-type phage and an amplification product, smaller by about 2 to 3 kb, for the phage deletion mutants D and E (Bruttin and Brüssow, 1996). Partial sequencing of these PCR products from several independent phage mutants revealed identical deletion sites: nucleotide (nt) position 3418/19 (left deletion site) and nt position 5809/10 (right deletion site; Fig. 1). The deletion was flanked at both sides by conspicuous direct and inverted DNA repeats. Within the 150-nt region surrounding the left deletion site four 5-nt repeats (TAAAA) are followed by a further four 5-nt repeats (TTTTA), which are the inverted repeats of the first. In addition, one 10-nt-long inverted repeat showing one mismatch was identified (Fig. 1). Within 300 nt of the right deletion site two distinct direct repeats, one 10 and another 16 nt long, were observed. In addition, two different 11-nt-long inverted repeats were detected. No homology could be found between the DNA sequences, including the repeats, at the left and right deletion sites.

Within the analyzed DNA fragment, such a concentration of DNA repeats was rather specific to the deletion sites. Two other areas of the analyzed phage DNA possessed notable DNA repeats: 20- and 26-nt-long direct repeats preceded by 12- and 13-nt-long inverted repeats, respectively, were detected between nucleotide positions 1430 and 1535, and 1995 and 2417 (Fig. 1).

Prediction of open reading frames and homology searches

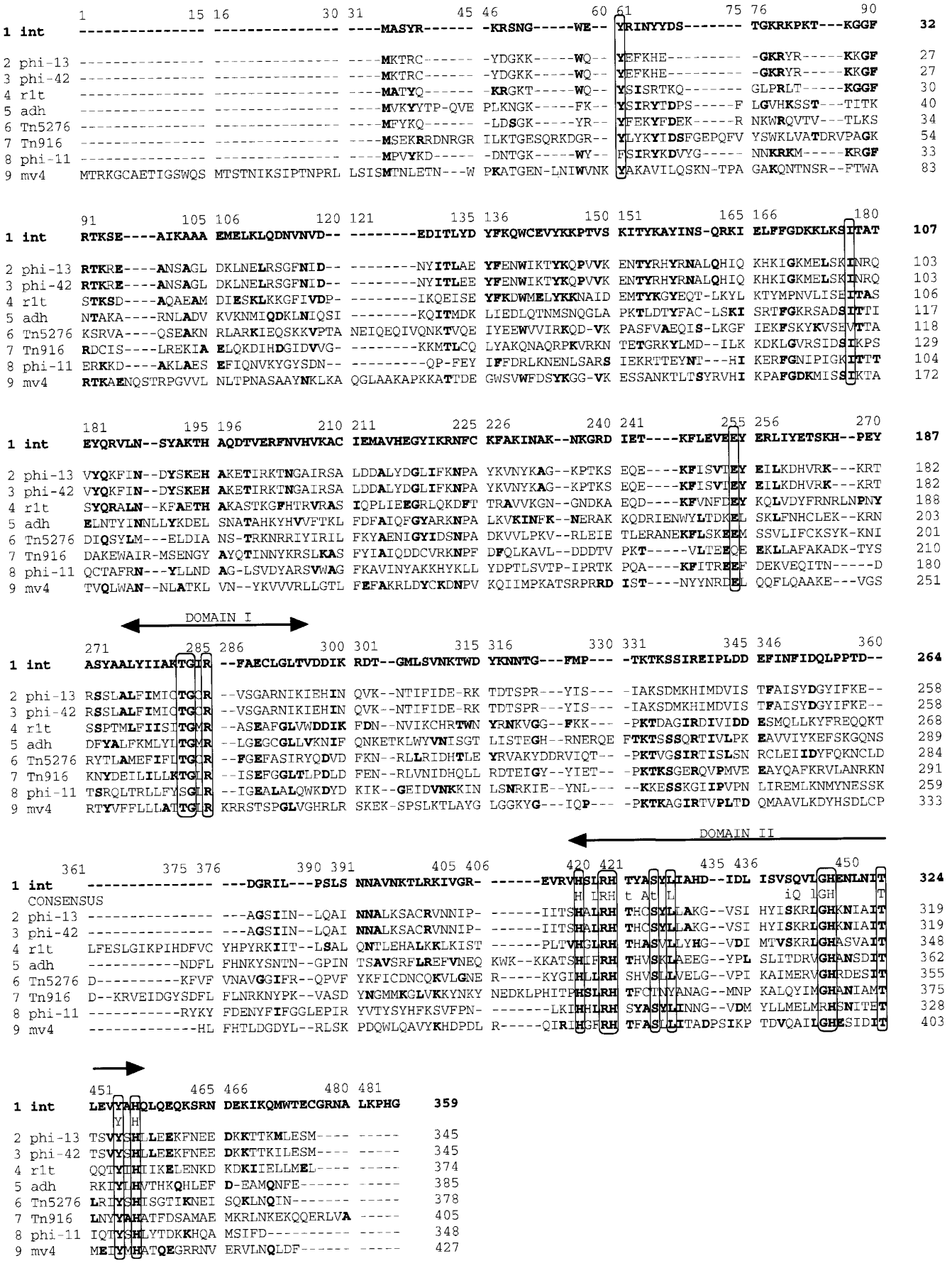
The sequenced DNA and the adjacent *Xba*I fragment reported previously (Bruttin and Brüssow, 1996) were investigated for open reading frames (orf) longer than 50 codons and using ATG start codons. Nineteen orfs potentially coding for proteins ranging from 54 to 359 aa were detected (Fig. 1). The orfs were labeled according to the number of aa in the predicted protein. Only 7 orfs were preceded by a standard *S. thermophilus* ribosomal binding site (GAG; Guédon *et al.,* 1995) in appropriate spacing with respect to the start codon. It should be noted that the GAG consensus is based on relatively few sequences of *S. thermophilus,* but not of its bacteriophages. Even within the *eps* gene cluster of *S. thermophilus* only 9 of 14 genes are preceded by the standard GAG sequence (Stingele *et al.,* 1996). Analysis of the orfs revealed that between nt positions 1 and 3250 the majority of the predicted orfs pointed to the right, between nt positions 3250 and 6100 all but one predicted orf pointed to the left (i.e., they were transcribed from the opposite strand), while all orfs between nt positions 6200 and 8100 pointed again to the right.

The orfs were translated into a protein sequence and the databases were screened for homologous proteins with the BLAST P program. Possible functions could be attributed to several orfs (for details see below under the specific subheadings). orf 359 demonstrated a high degree of homology to the integrase family of proteins. The adjacent orf 203 showed homology to an anonymous orf 258 from the temperate lactococcal phage BK5-T. Notably, orf 258 was also found adjacent to the *int* gene from BK5-T (Boyce *et al.,* 1995). orfs 122, 127, and 75 demonstrated weak to moderate homology with several phage and bacterial repressors, while orf 287 showed a good homology to an antirepressor of phage P1 as reported previously (Bruttin and Brüssow, 1996). In the left part of the analyzed DNA fragment orf 87a showed homology with holins and orf 288 showed weak homology with orf 259 from lactococcal ϕBK5-T.

Definition of the module

According to the DNA homology searches the contiguous DNA fragment between nt positions 3400 and 7200 represents functions essential for the regulation of the lysogenic life style of ϕSfi21. As the putative holin is a gene function common to lytic and temperate phages, we suspected the transition between the putative lysogeny and lysis modules somewhere between nt positions 500 and 3400. To define the borders of the lysogeny module, Southern blot hybridizations were done using defined ϕSfi21 DNA fragments as probes against 26 lytic phages covering the whole range of our phage collection. When the *Xba*I fragment 4 (nt positions 5590 to 8130) was used as a probe, only ϕSfi21, meaning none of the 26 lytic phages hybridized (Fig. 2B), indicating that orf 87 b (ending at nt 7830) belonged to the lysogeny module. According to comparative Southern blot hybridizations the left side of the ϕSfi21-specific DNA extended to orf 110 (starting at nt 1759) (Fig. 2B). The transition from ϕSfi21-specific DNA to DNA shared between the temperate and lytic phages occurred between orf 288 and orf 110. The transition point was in fact very sharp as a probe covering nt positions 1269 to 1564 hybridized with 22 of 26 lytic phages (Fig. 2C), while a probe covering nt positions 1564 to 2219 hybridized exclusively with phage ϕSfi21 (data not shown). The left border of the lysogeny module was preceded by a 20-nt-long direct repeat and an 11-nt inverted repeat.

FIG. 3. Multiple sequence alignment of the deduced product of ϕSfi21 orf 359 and various integrases. Amino acids identical to the ϕSfi21 integrase are in bold. The integrases shown are from *Staphylococcus aureus* phages phi-11 (Accession No. M34832), phi-42 (U01872), and phi-13 (X82312); *Lactobacillus gasseri* phage adh (M62697), *Lactobacillus delbrueckii* phage mv4 (P20710), *Lactococcus lactis* phage r1t (U38906) and transposon *Tn5276* (L27649), *Enterococcus faecalis* transposon Tn916 (M37184). Amino acids that are conserved in at least 8 of the 9 sequences are boxed. Domains I and II represent conserved motives of integrase and resolvase proteins.


DOMAIN I
DOMAIN II

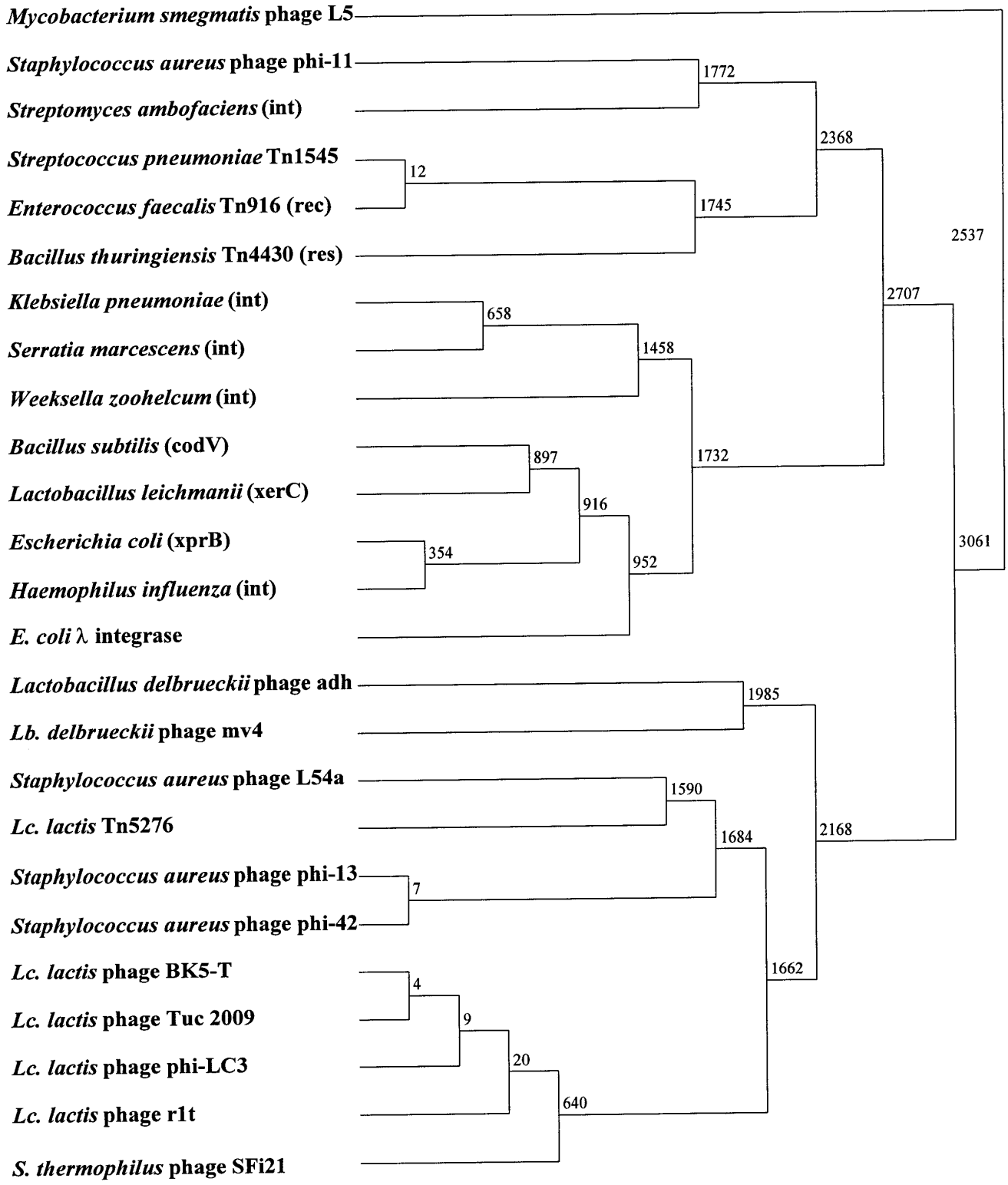


FIG. 4. Tree analysis of ϕSfi21 integrase and indicated integrases of various bacterial origin. The tree was built using the ALIGNMENT program of GeneWorks (V2.0) from Intelligenetics Inc. The numbers show the cost of the alignment. Lb, *Lactobacillus;* Lc, *Lactococcus;* S, *Streptococcus.*

A further DNA probe covering orf 87 (holin) and 288 hybridized with 13 of 26 lytic phages (Fig. 2D). With this probe only phage Sfi18 showed an identical hybridization pattern to phage Sfi21.

Integrase

Alignment of the ϕSfi21 *int* homologue (translation of orf 359) with different members of the *int* gene family

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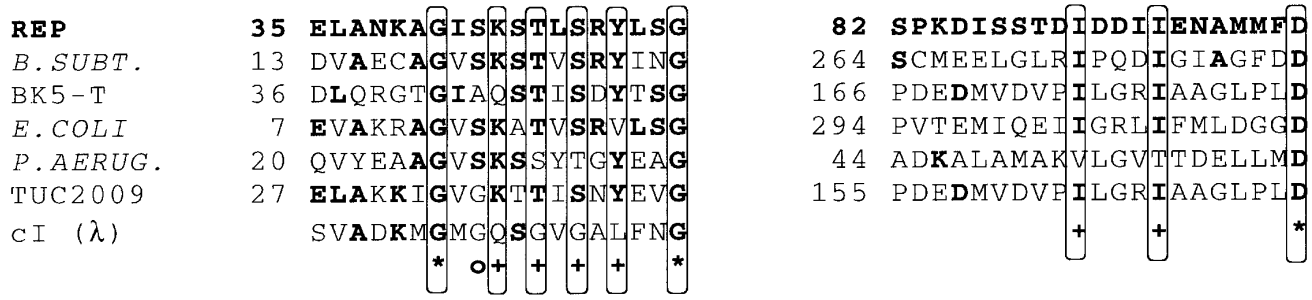

FIG. 5. Alignment of the helix-turn-helix motif (left) and the RecA cleavage site (right) found in the ϕSfi21 cI homologue (REP, upper line in bold) with cI-type repressors from *Lactococcus lactis* phages BK5-T (L44593) and Tuc2009 (L26219), *Bacillus subtilis* catabolite repressor CcpA (L47838), *E. coli asc* operon repressor (P24242), and transcription repressor of *Pseudomonas aeruginosa* (L06240). Bottom left, the corresponding sequence from the cI protein of phage λ is shown. Amino acids identical to the ϕSfi21 cI homologue are shown in bold. *Conserved in all sequences; + conserved in four other sequences; °conserved in three other sequences. Amino acid positions conserved in all but one sequence were boxed.

(Fig. 3) showed a close relationship with the integrases of *Lactococcus lactis* bacteriophage r1t (30.6% aa identity; van Sinderen *et al.,* 1996) and *Staphylococcus aureus* bacteriophage phi-42 (26.5%; Carroll *et al.,* 1995). More than 20% aa identity was found with integrases from *Lactobacillus delbrueckii* subsp. *bulgaricus* bacteriophage mv4 (22%; Dupont *et al.,* 1995), *Lactobacillus gasseri* bacteriophage adh (23.4%; Raya *et al.,* 1992) and *Lactococcus lactis* transposon Tn5276 (20.3%). 17.5% aa identity was found in comparison with integrases from the *Enterococcus faecalis* transposon Tn916 and the *Staphylococcus aureus* bacteriophage phi-11. Homology was not restricted to the conserved domains 1 and 2, but was scattered throughout the entire protein sequence (Fig. 3).

Tree analysis of integrases showing homology with ϕSfi21 Int defined three major branches represented by *Mycobacterium smegmatis* phage L5 (Lee *et al.,* 1991), phage λ, and a branch to which ϕSfi21 Int belonged (Fig. 4). A subbranch of the third group was composed exclusively of *Lactococcus lactis* phage integrases and the integrase from our temperate *S. thermophilus* phage. A sister subbranch to ϕSfi21 Int was represented by *Staphylococcus aureus* phage integrases and somewhat more distant, but still belonging to the third branch, were integrases from *Lactobacillus delbrueckii* phages. No systematical family attribution could be made for the integrases belonging to the second branch, where phage λ integrase was a member; e.g., it covered integrases from gram-positve and gram-negative organisms.

Repressor

The predicted protein from orf 127 showed weak homology (15% aa identity, $P=0.0029$) with the cI-like putative repressor of the temperate lactococcal phages Tuc2009 (van de Guchte *et al.,* 1994). Orf 127 showed two clear repressor motives. We identified a helix-turn-helix motif showing homology with phage λ cI repressor (Pabo, 1992; Fig. 5A). In addition, a motif showing similar-

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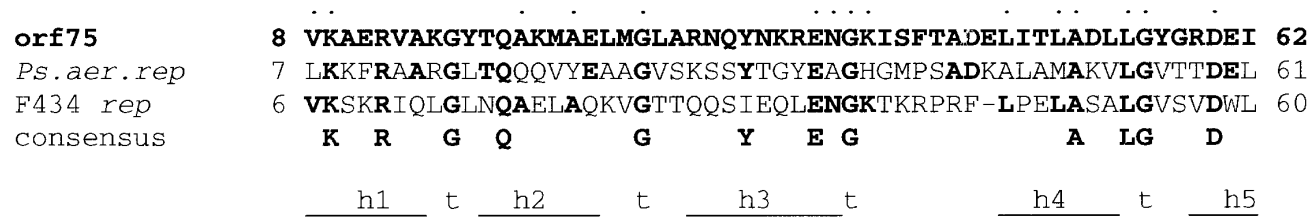

FIG. 6. Alignment of a 55-aa stretch predicted for orf 75 from ϕSfi21 with a *Pseudomonas aeruginosa* bacteriophage transcription repressor (Accession No. L06240) and cI repressor from the lambdoid phage 434 (Y00118). Amino acid positions conserved between orf 75 gp and phage 434 repressor were marked by a dot above the alignment; amino acid positions conserved in all three sequences were given in the consensus. The helix (h)-turn (t)- helix (h) motifs were given for phage 434 repressor (Mondragon *et al.,* 1989). Note the absolute conservation of the glycine in all the turn positions.

7

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                1              15 16             30 31             45 46             60 61             75 76             90
SFi21 orf203 ---------------- ---------------- ---------------- MKDEKKVLGILAIVF GGISLTISWVPILNN VS--FYIAVISLILG  43
BK5-T orf258 MKINTIQVLIASTLA LGTVALTQDVKAADQ TTSIYRLYNKHTGEH FYTKSHTEQLNAIMA GWDDEGTGWVAPLSS NSPVYRVYNPNATGG  90
                                                                **   *        **  *    *           *

                91          105 106          120 121          135 136          150 151          165 166          180
SFi21 orf203 VIALIVNRKNKKVLA IVGTCLSVAS--III VLVTQSMYSSAIDNA SKAIDKASSSIDAEY SFVQKNGTYVVDSKT GSGLK    203
BK5-T orf258 DHYYTKSKYEAQSLV NRGWKWDYNGQPVFY SGGNSSVYVAYNPNA QSGSHNYTMNSFEQN SLLNNGWKYGATAWN AVTQFNWTLNQYKSL 180
                      *    *                * *     **                *                *   * **     *

                181         195 196          210 211          225 226          240 241          255 256          270
SFi21 orf203 VDG------TTTYDE IVATVGEPNKTETDT DYDIDTDSKVPSMDC DWDLEDGSYYASVSI HFVQKNGTYVVDSKT GSGLK    203
BK5-T orf258 VVGDSNGNGGTNYNS VLASHDIP-TDITSF STDGYASKTVIYNNT NWDYSDGNYKSVVLT FIKQANGSYLLGYKN YINL-    258
                *          * *     *    *    *     *       *      **  **  *   *      * ** *    *     *
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FIG. 7. Alignment of the predicted protein of orf 203 from ϕSfi21 with that of orf 258 from lactococcal phage BK5-T (Accession No. L44593). * Symbolizes identical aa.

ity with the RecA cleavage site (Sauer *et al.,* 1982) homology region of the lactococcal phage repressors was identified, although the Ala/Gly cleavage site was not preserved (Fig. 5B). One should note, however, that the *E. coli* and *B. subtilis* repressors also did not possess this Ala/Gly site. Interestingly, the *S. thermophilus* phage cI homologue was half the size of its lactococcal homologue (Tuc2009, 286 aa) or of the phage λ cI repressor (236 aa).

Croequivalent and antirepressor

The lytic/lysogenic life cycle decision in phage λ is realized by a competitive interplay between the cI repressor and the Cro protein for operator binding. The operators are situated between the *cI* and *cro* genes, which are adjacent to each other, but are transcribed in opposite directions (Gussin *et al.,* 1983). A similar control circuit is found in lactococcal phage r1t (Nauta *et al.,* 1996). In phage SFi21 the topological homologue of *cro* is orf 75 (Fig. 1). The predicted protein product of orf 75 showed homology to the N-terminal part of the cI repressor protein of the lambdoid phage 434 (Fig. 6), namely the helix-turn-helix motif with which this protein binds DNA (Modragon *et al.,* 1989). A similar degree of homology was seen with a putative DNA binding transcription repressor of a *Pseudomonas aeruginosa* phage (Fig. 6). The observation of direct repeats between orf 127 and orf 75 (Fig. 1), and the prediction of DNA binding activity for both proteins, may identify this DNA region by analogy with other phage systems as the genetic switch region of phage SFi21. The topological equivalent of *cro,* orf 75, overlaps orf 287, which is in a different reading frame. The predicted protein product of orf 287 showed good homology to the antirepressor of phage P1 (Bruttin and Brüssow, 1996) and thus may also be involved in the lytic/lysogenic life cycle decision.

Orf 203 and phage resistance

The predicted protein product of orf 203 showed 21.7% aa identity with that of orf 258 from lactococcal phage BK5-T (Boyce *et al.,* 1995; Fig. 7). This homology was further underlined by an identical position in the phage genome since in both cases the putative gene was situated between the *int* and the putative *cI* genes. A gene of unknown function situated between *int* and *cI* genes was also described for the lactococcal phage r1t (van Sinderen *et al.,* 1996). Orf 203 was of interest to us since we have observed a phage resistance phenotype associated with the plasmid pPX3R, possessing the phage Sfi21 *int* gene and orf 203.

When our indicator *S. thermophilus* strain Sfi1, which is susceptible to 21 distinct phage strains, was transformed with the plasmid pPX3R we observed a significant reduction in the efficiency of plaquing (e.o.p. $\leqslant 10^{-3}$) for 12 of the 21 phages compared to the vector control (Table 1). In some cases the exact titer reduction was difficult to evaluate as the phage plaque size was substantially reduced on Sfi1 cells transformed with plasmid pPX3R. The pattern, but not the strength, of phage resistance conferred by this plasmid was similar to that conferred by the prophage ϕSfi21 in the lysogenic Sfi1 host (Table 1). Phages ϕST28, ST25, ST44A, ST33, ST17, Sfi3J, Sfi19, and S17 were only incompletely or not at all inhibited by the ϕSfi21 prophage in the lysogenic Sfi1 host, which thus defined phages belonging to a different immunity group. Interestingly, phages ϕST28, ST25, ST44A, ST17, Sfi3J, Sfi19, and S17 were also incompletely inhibited (e.o.p. $\geqslant 10^{-3}$) by the plasmid pPX3R. A notable difference to the lysogen-mediated immunity function was that the plasmid pPX3R did not protect Sfi1 cells against infection with homologous ϕSfi21 or its deletion derivative, ϕS3. It should be noted that a totally different pattern of phage resistance was observed when an anonymous 4-kb DNA fragment from the lytic phage ϕS17 was introduced into the Sfi1 host (data not shown). To demonstrate further the specificity of the phage resistance conferred by the plasmid pPX3R, several other DNA fragments from the temperate ϕSfi21 were introduced into the Sfi1 host and failed to confer a protection against phage infection (data not shown).

In order to pinpoint the factor responsible for the ob-

TABLE 1

Titration of the Indicated *S. thermophilus* Phages on Normal Sfi1 Host and Its Lysogenic Derivative (Sfi1.cl.6) and Sfi1 Cells Transformed with Plasmids pNZ124 and pPX3R

Phage	SFi1	SFi1.cl6	pNZ124	pPX3R	pMZ27
ϕM4-15	5×10^4	<10	5×10^5	<10	1×10^5
ϕM4-31	4×10^4	<10	1×10^6	<10	7×10^5
ϕST28	9×10^8	9×10^3	9×10^8	2×10^6	5×10^8
ϕST25	9×10^8	2×10^5	3×10^8	2×10^6	2×10^8
ϕST42	10^8	<10	2×10^9	1×10^5	2×10^9
ϕST44	2×10^8	<10	2×10^9	1×10^6	1×10^9
ϕST44A	8×10^8	8×10^9	4×10^8	2×10^6	5×10^6
ϕST33	2×10^9	6×10^4	3×10^8	1×10^5	5×10^6
ϕST17	3×10^9	3×10^6	7×10^9	5×10^7	2×10^9
ϕST3	10^9	<10	2×10^9	2×10^6	8×10^8
ϕS96	2×10^8	<10	2×10^6	<10	<100
ϕSFi3J	2×10^9	4×10^{10}	9×10^8	1×10^7	8×10^7
ϕSFi19	2×10^9	2×10^7	3×10^9	1×10^8	2×10^8
ϕS89	5×10^8	<10	2×10^8	<100	3×10^7
ϕS19	4×10^7	<10	6×10^7	<100	2×10^6
ϕS69	2×10^8	40	6×10^7	$<10^3$	4×10^7
ϕSFi18	9×10^7	<10	2×10^8	<100	6×10^8
ϕS3	2×10^8	<10	8×10^8	2×10^7	$<10^3$
ϕSFi21	4×10^9	<10	2×10^8	2×10^{6a}	4×10^8
ϕSFi2	8×10^7	<10	4×10^9	5×10^4	2×10^9
ϕS17	6×10^9	5×10^9	4×10^7	5×10^5	<10

Note. The phage titrations (given as PFU/ml) on Sfi1 and Sfi1.cl6 on the one hand and Sfi1 host transformed with plasmids pNZ124 and pPX3R on the other hand were done at different times with different phage stocks. Titer reductions by a factor of 1000 are underlined.

[a] Pinpoint plaques at the limit of detection.

served bacteriophage resistance, deletions were generated in the *int* gene and orf 203. The 498-bp *Bfr*I–*Eco*RV fragment was deleted from pPX3R, yielding the clone pSF101, in which the *int* gene was disrupted. Subsequent digestion of pSF101 with *Cla*I and *Nsp*V, followed by religation, resulted in the construct pSF45, which possessed a deletion of 212 bp within orf 203. When these clones were tested in the Sfi1 bacterial host we could assign the phage inhibitory action to orf 203 (Table 2). The strength of this inhibitory action was also tested by chromosomal integration of the PX3R DNA fragment in Sfi1. The resulting strain was bacteriophage sensitive (Table 2), indicating that orf 203 is not effective as a single copy.

Holin

Orf 87a at the 5′ end of the sequenced area showed significant homology with holins from two lactococcal phages, Tuc2009 (Arendt *et al.,* 1994) and phi-LC3 (Birkeland, 1994) (34.5% aa identity). The aa identity was even higher (36.8%) when compared with *Staphylococcus aureus* phage phi-11 (Weerakoon and Jayaswal, 1995) (Fig. 8). A very lysine-rich N-terminus was observed for the putative ϕSfi21 holin but this was not the case for the other holins.

The adjacent orf 288 showed homology to orf 259 from lactococcal ϕBK5-T: over a 35-aa stretch in the N-terminal half of the predicted ϕBK5-T protein 12 identical positions were seen in the protein predicted for ϕSfi21 (Fig. 9). In several lactococcal phages the lysin gene was found adjacent to the holin gene (Arendt *et al.,* 1994; Birkeland, 1994); however, orf 288 showed no homology to phage lysins.

DISCUSSION

Since loss and gain of modules are a major driving force for phage evolution (Casjens *et al.,* 1992; Susskind and Botstein, 1978), elucidation of the underlying mechanisms would be of substantial interest. As the 2.4-kb site-specific deletion in phage ϕSfi21 was flanked at both sites by conspicuous repeats, we tested whether the deletion process could be the result of a site-specific recombinase responsible for module shuffling, which in this case effected the transformation of a temperate into a lytic phage. If the observed deletion is a consequence of a hypothetical recombinase implicated in module-shuffling, one might expect the deletion to define a functional module. The definition of the lysogeny module and a closer examination of the DNA repeat sequences should elucidate this.

The data presented in this report demonstrated that the putative lysogeny module comprises at least 6.6 kb of genetic information. The length estimation of the lysogeny module was based on Southern blot hybridization experiments which differentiated ϕSfi21-specific sequences (approximate nt positions 1550 to $\geqslant$8100) from sequences shared with lytic phages (nt positions 1 to 1550). ϕSfi21-specific DNA sequences do not necessarily define lysogenic functions, but a minimal extension of the lysogeny module is given by the attribution of likely functions to the identified orfs by database searches. Between nt positions 3400 and 7200, sequence analysis identified genes which could potentially encode for an

TABLE 2

Titration of *S. thermophilus* Phage S89 on Normal Sfi1 Host and Sfi1 Cells Transformed with the Indicated Plasmids

Host	Phage titer (PFU/ml)
Sfi1	3×10^8
Sfi1 pNZ124	7×10^7
Sfi1 pPX3R	<100
Sfi1 pSF101	<100
Sfi1 pSF45	1.6×10^8
Sfi 2.8[a]	4×10^8

[a] Sfi 2.8 is Sfi1 containing an integrated copy of PX3R DNA.

8

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          1              15 16            30 31            45 46            60 61            75 76            90

hol       MKKRKKKMINFKLRL QNKATLVALISAVFL MLQQFG--------- LHVPNNIQEGINTLV GILVILGIITDPTTK GIADSERALSYIQPL
LysA      MN-----QINWKLRL KSKAFWLALLPALFL LIQAIGASFGYKWNF VILNQQLAAVVNAAF ALLAIVGVVADPTTS GLGDSDRVLNKDKSE
Tuc 2009  MN-----QINWKLRL KSKAFWLALLPALFL LIQAIGAPFGYKWDF VILNQQLAAVVNAAF ALLAIVGVVADPTTS GLGDSDRVLNKDKSE
phi-11    MD------INWKLRF KNKAVLTGLVGALFV FIKQVTDLFG--LDL STQLNQASAIIGAIL TLLTGIGVITDPTSK GVSDSSIAQTYQAPR
          *       ** ***    **    *  * *                                    *   *   ***   *  **
          91           105 106          120 121          135 136          150 151          165 166          180

hol       DDKEVY  96
LysA      ENK---
Tuc 2009  ENK---
phi-11    DSKKEE
            *
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FIG. 8. Alignment of the predicted protein of orf 87a from ϕSfi21 with holins from *Staphylococcus aureus* phage phi-11 (Accession No. L34781), lactococcal phage Tuc2009 (L31364), and LysA holin-like protein from lactococcal phage phi-LC3 (U04309). The conserved aa are indicated in bold. * Symbolizes identical aa in all four sequences.

integrase, a cI-like repressor, a Cro-like protein, and an antirepressor. Furthermore, the distribution and orientation of orfs in phage ϕSfi21 were remarkably similar to that in the lactococcal phage BK5-T (Boyce *et al.,* 1995).

The noticeable repeats which flank the type 2 deletion are unlikely to represent recognition sites for a module-recognizing recombinase. Immediately downstream of the left deletion site we detected the *int* gene with the 3′-end of the gene pointing to the deletion site. Classically the *attP* site is found directly downstream of the *int* gene (Carroll *et al.,* 1995). In bacteriophages from lactic acid bacteria the *attP* site is typically surrounded by a complicated array of direct and inverted repeats (Boyce *et al.,* 1995; Dupont *et al.,* 1995; Lillehaug and Birkeland, 1993; van de Guchte *et al.,* 1994). The repeats at the left side of the deletion are thus not recognition elements for a hypothetical module recombinase, but likely recognition elements for prophage integration. The right deletion site was also flanked by conspicuous DNA repeats which are most likely genetic elements involved in the genetic switch between the lytic and lysogenic modes of phage infection (see below). The type 2 deletion in phage Sfi21 is thus probably due to an Int-mediated cross-over event between the phage *attP* site and a secondary *att* site in a nonessential region of the phage genome. For a lytic phage derivative of phage Sfi21 any DNA region within the lysogeny module becomes necessarily a nonessential region. Similar deletion mutants have been isolated for phage λ by Davies and Parkinson (1971). Many deletions of heat-resistant phage λ mutants had one end point at the phage *attP* site and another more variable endpoint in secondary *att* sites. Current work in our laboratory confirms that the left deletion site of the type 2 spontaneous phage deletion mutants is identical to one end of the core sequence shared between the *attB* and *attP* sites (manuscript in preparation).

A phage inhibitory activity was associated with plasmid pPX3R and its derivatives containing an intact orf 203 from ϕSfi21. The orf 203-mediated activity showed similarity to the resistance of the lysogenic host against superinfection with heterologous phages. As no *cI*-like gene was present on this plasmid we may have identified a second phage repressor system in ϕSfi21, which is independent of the cI-like repressor. This phage repressor is, however, not efficient against the homologous ϕSfi21 and is only effective against heterologous phages when present in the multicopy state.

In phage λ, the cI repressor is counter-acted by the Cro protein, a small one-domain protein of about 70 aa in length with DNA binding activity. The *cro* gene is found adjacent, but in the opposite orientation to the *cI* gene. A set of operators to which cI and Cro bind is found between the *cI* and *cro* genes (Gussin *et al.,* 1983). A similar control circuit was observed in the lactococcal phage r1t (Nauta *et al.,* 1996). Orf 75 in ϕSFi21 is the topological equivalent of *cro.* As the gene products from both orf 127 and 75 showed DNA binding motifs and as a region with DNA repeats was situated between both

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Sfi21 orf288a   103 SGGAGGHVGVMMDSVNFISCDYSTQGAAGQAAINTY 137
                    S G+ GH G+ + + +FI C Y+  G A    + Y
BK5-T orf259     88 SAGSDGHTGIFLSNGSFIHCSYTHNGIAVDTNDAY 122
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FIG. 9. Alignment of a 35-aa stretch predicted for orf 288a from ϕSfi21 with that predicted for orf 259 from lactococcal phage BK5-T (Accession No. L44593). Identical amino acids are indicated in bold; + represents amino acid similarity.

genes, we might have identified the genetic switch region of φSfi21. Orf 75 overlaps the *ant* gene, potentially coding for an anti-repressor, in the same orientation but in a different reading frame. Therefore one might speculate on the possibility of a bipartite immunity system for the control of lysogeny in φSfi21.

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